

## Hydration and Protein Folding in Water and in Reverse Micelles: Compressibility and Volume Changes

D. Valdez,\* J.-Y. Le Huérou,\* M. Gindre,\* W. Urbach,<sup>†</sup> and M. Waks\*

\*Laboratoire d'Imagerie Paramétrique, UMR 7623 CNRS Université Pierre et Marie Curie, 75270 Paris cedex 06, France; <sup>†</sup>Laboratoire de Physique Statistique, UMR 8550 CNRS Ecole Normale Supérieure, 75231 Paris cedex 05, France

**ABSTRACT** The partial specific volume and adiabatic compressibility of proteins reflect the hydration properties of the solvent-exposed protein surface, as well as changes in conformational states. Reverse micelles, or water-in-oil microemulsions, are protein-sized, optically-clear microassemblies in which hydration can be experimentally controlled. We explore, by densimetry and ultrasound velocimetry, three basic proteins: cytochrome *c*, lysozyme, and myelin basic protein in reverse micelles made of sodium bis (2-ethylhexyl) sulfosuccinate, water, and isooctane and in aqueous solvents. For comparison, we use  $\beta$ -lactoglobulin ( $pI = 5.1$ ) as a reference protein. We examine the partial specific volume and adiabatic compressibility of the proteins at increasing levels of micellar hydration. For the lowest water content compatible with complete solubilization, all proteins display their highest compressibility values, independent of their amino acid sequence and charge. These values lie within the range of empirical intrinsic protein compressibility estimates. In addition, we obtain volumetric data for the transition of myelin basic protein from its initially unfolded state in water free of denaturants, to a folded, compact conformation within the water-controlled microenvironment of reverse micelles. These results disclose yet another aspect of the protein structural properties observed in membrane-mimetic molecular assemblies.

### INTRODUCTION

Although the interiors of folded proteins, or protein domains, are compact and well-packed (Klapper, 1971; Richards, 1977; Richards and Lim, 1994), the packing of amino-acid residues is not perfect (i.e., proteins are compressible). In addition to imperfect packing, a significant fraction of protein compressibility originates from the finite rigidity of the intermolecular interaction potentials. Gekko and Nogushi (1979) pointed out that, in addition to imperfect packing, the presence of internal cavities (Rashin et al., 1986) contributes positively to protein adiabatic (isentrope) compressibility as well as volume changes. When such voids are large enough to accommodate water molecules, internal water can then act as a structure stabilizer (Takano et al., 1997) by maintaining good hydrogen bonds between the domains and filling sites of imperfect packing.

As a universal solvent, water interacting at the protein surface contributes negatively to protein volume and compressibility. Difficulties in interpreting the precise changes in protein volume and compressibility induced by hydration at protein-water interfaces have led to empirical estimates of the intrinsic protein volume, that is, the volume that cannot be penetrated by the solvent (Paci and Velikson, 1997). There is no consensus on the exact value of the compressibility of the solvent-inaccessible protein core, or intrinsic protein compressibility, due to the diverse approaches used by different investigators (Gekko and Noguchi, 1979;

Gavish et al., 1983; Kharakoz and Sarvazyan, 1993; Chalikian et al., 1996).

Recent developments in acoustic techniques (difference ultrasound velocimetry) have rendered possible high precision compressibility measurements of small volumes of solutes, in particular biopolymers (Sarvazyan, 1991). Thus, the experimental study of protein volume and adiabatic compressibility variation as a function of increasing levels of hydration should become possible and offer unique insights into these observations.

Experiments designed to address the latter issue cannot be performed in a traditional solvent such as water in the bulk state, a medium in which investigations have previously been carried out. We have therefore used a non-conventional solvent designated as microemulsions, or reverse micelles, in this work. Reverse micelles can be described as water microdroplets dispersed in water-immiscible apolar solvents (i.e., oils) and stabilized by a monolayer of surfactant acting as an interface between oil and water. The surfactant non-polar tails protrude into the oil, while the polar headgroups are in direct contact with the central water core. An important feature of the anionic surfactant sodium bis (2-ethylhexyl) sulfosuccinate (AOT) is the tight binding by one polar headgroup of about 7 to 10 water molecules. There is a wealth of information available concerning reverse micelles, such as their structure, their phase behavior, and the physical properties of the surfactant micellar bound and free waters (Luisi and Magid, 1986; Oldfield, 1994; Amararene et al., 2000).

The size of the droplets, which are in thermal equilibrium, depends only on the water concentration. As water is added, their radius (range, 1.5 to 10 nm) increases as a function of the water:surfactant molar ratio ( $W_0$ ). (Amararene et al., 2000). The water content of the system can

Received for publication 28 June 2000 and in final form 19 March 2001.

Address reprint requests to Dr. Marcel Waks, LIP, UMR 7923 CNRS, Université Pierre et Marie Curie, 15, Rue de L'Ecole de Médecine, Paris, France 75006. Tel: 33144414976; Fax: 33146335673; E-mail: marcel.waks@lip.bhdc.jussieu.fr.

© 2001 by the Biophysical Society

0006-3495/01/06/2751/10 \$2.00

thus be precisely varied and experimentally controlled. Reverse micelles are protein-sized and, consequently, can accommodate proteins within their water cores. Since micellar solutions are optically clear, all current spectroscopic measurement techniques (e.g., absorption, fluorescence, and circular dichroism) are available. Therefore, the protein concentration, solubility and conformation can be determined with high precision.

We have undertaken this work to explore the effect of hydration on the specific volume and compressibility of proteins sequestered in the micellar aqueous core. For this purpose, we have selected three basic proteins of known structure in aqueous solution and in micellar solutions: cytochrome *c*, lysozyme and myelin basic protein. Due to their positive charge they experience a strong electrostatic field in AOT reverse micelles. For comparison, we have also carried out measurements with  $\beta$ -lactoglobulin ( $pI = 5.1$ ). As in water, adiabatic compressibility is more sensitive in reverse micelles to protein hydration than volume. In all the proteins examined, we observe a decrease in compressibility as a function of hydration, with the exception of myelin basic protein (MBP). At the lowest water concentration, compatible with total protein solubilization, we report large compressibility values in the range of those of a protein interior.

Moreover, the same experimental strategy has allowed us to obtain interesting volumetric data on MBP, a peripheral membrane protein from the nervous system. In water free of denaturing agents, the protein displays a high degree of conformational flexibility with little periodic structure (Liebes et al., 1975). In the aqueous core of reverse micelles, however, MBP folds into a well-ordered, compact periodic structure (Nicot et al., 1985). We have taken advantage of this unique structural property to characterize both the aqueous unfolded and the micellar folded conformational states of MBP. The controlled micellar microenvironment, significantly different from bulk water, appears to mirror different aspects of complex cellular conditions, and serves as a model of the myelin interlamellar space (Nicot and Waks, 1985; Waks, 1986), in which MBP is located in myelin.

## MATERIALS AND METHODS

### Materials

AOT purchased from Sigma (SIGMA ULTRA) was 99% pure and used after sufficient desiccation in vacuum over phosphorus pentoxide (SICA-PENT from Merck). The nonionic surfactant, tetraethylene glycol monododecyl ether ( $C_{12}E_4$ ), was obtained from Nikko Chemicals (Japan), and judged to be > 99% pure from gas chromatography. Water-free isooctane, Pro Analysis grade, was purchased from Merck, while decane > 99% pure came from Sigma. Water used in this study was of MILLIQ purity (pH around 6). The proteins were from Sigma: hen egg lysozyme (L 6876), heart muscle cytochrome *c* (C 7752) and bovine milk  $\beta$ -lactoglobulin (L 3908). After dialysis against cold water and lyophilization, they were

carefully dried in vacuo. MBP was prepared and purified from bovine brain, as has been previously described (Nicot et al., 1985).

### Sample preparation

All the samples were prepared by first weighing, with a precision of  $\pm 0.03$  mg, the dried, lyophilized proteins on a Model 1712 Sartorius balance, in 20 ml volumetric flasks (class A  $\pm 0.04$  ml). The solvents used to make up the volume at 20°C were solutions of the surfactants (0.1M AOT in isooctane or 0.15 M  $C_{12}E_4$  in decane) with the appropriate amount of water required to achieve the desired water:surfactant molar ratio. For all proteins used, a precise solubility curve (concentration versus  $W_o$ ) was established by optical density at 280 nm, since extinction coefficients are identical in water and in reverse micelles (Luisi and Magid, 1986). Volumetric experiments were always carried out well below the solubility limit. After several minutes of gentle shaking and a few seconds of sonication, optically clear solutions were ready for use.

Note that proteins are not soluble in isooctane, a water immiscible solvent. We have been unable to detect traces of protein in the organic solvent by fluorescence spectrophotometry. Experiments under conditions in which protein would be only partially solubilized and remain suspended in the oil (i.e., turbid samples detected by optical density at 350 nm) were ruled out, as they would not have been optically transparent. The conformational properties of the above proteins in reverse micelles have been reviewed, and they do not denature during the solubilization process (Nicot and Waks, 1995). By systematically applying this procedure, we have found limits of solubilization for each protein studied as a function of  $W_o$  in relation to their respective size and surface charges. This fact is clearly illustrated in Figs. 1 and 2. For example, while  $\beta$ -lactoglobulin cannot be completely solubilized at a working concentration ( $\sim 3$  mg/ml) below  $W_o = 10$ , cytochrome *c* remains partially insoluble at  $W_o$  values above 22. In contrast to the other proteins, MBP displays a solubility maximum at  $W_o = 5.6$  due to its high affinity for membrane-bound water (Nicot and Waks, 1985). During all the experiments the greatest care has been taken to avoid evaporation of the organic solvent. The samples were always kept in ground glassware and transferred without delay to the densitometer and the air-tight ultrasound measurement cells (see below) by the means of glass syringes.

### Volumetric measurements

The densities of micellar solutions and protein-containing micellar solutions were determined at  $25.00 \pm 0.01^\circ\text{C}$  using a vibrating tube Anton Paar DMA 58 digital density meter standardized by water and dry air. The precision obtained on density is better than  $10^{-5} \text{ g}\cdot\text{ml}^{-1}$  for a single measurement. Each set of measurements was carried out at least five times, the results averaged, and the value then used to calculate the protein apparent specific volume  $\varphi_v$  (see below). The stability of the apparatus is crucial: no evaporation of solvent occurs during the measurement procedure. Obviously, evaporation would result in increasing protein concentration and density values, and consequently a systematic drift of the density meter, which we do not observe.

### Ultrasound velocity measurements

Several methods of ultrasound velocity determination have been described to date, for example, the time-of-flight measurement, the sing-around, resonance, and pulse overlap methods (Braezale et al., 1981). We have selected a method based on time-of-flight determination, which offers, in addition to its simplicity, a high precision provided by recent instrumental and computational advances (Le Hu  rou et al., Compressibility of nano inclusions in complex fluids by ultrasound velocity measurements. Submitted for publication).

## Principle

In ultrasound velocity measurements using the time-of-flight technique, a short electrical pulse is applied to a piezoelectric transducer, converting the electrical wave into an acoustical one which then propagates through the sample. A second transducer converts the received wave into an electrical signal, which is compared to the excitation signal. The time interval elapsed between the emitted and received signals combined with knowledge of the precise distance between the two transducers allows determination of the ultrasound velocity.

## Measurement system

One of the principal limitations of the custom-built system resides in possible temperature drifts. This difficulty is circumvented by the use of a set of two cells of identical acoustic path enclosed in a single metal thermostated block (Sarvazyan, 1982). Experiments are carried out at  $25.00 \pm 0.01^\circ\text{C}$ , in a temperature controlled room. The setup allows the sequential determination of the ultrasonic velocity difference between the reference and the measuring cell at identical temperature, achieving a considerable improvement in sensitivity. First, the two acoustic cells are filled with the protein-free micellar reference solution ( $\approx 3$  ml) using a glass syringe, and velocity measurements are carried out. In a second step, the measuring cell is drained, rinsed, dried, and refilled with the liquid under investigation (i.e., the protein-containing micellar solution). The cells are made airtight with Teflon o-rings to avoid any liquid leakage or solvent evaporation. The final precision in ultrasound velocity determination is better than  $10^{-5}$ . The experimental setup has been described in detail elsewhere (Le Hu  rou et al., Submitted for publication).

## Protein volume and compressibility in reverse micelles

The protein apparent specific volume is given by:

$$\varphi_v = \frac{1}{\rho_1} - \frac{\rho - \rho_1}{c_p \rho_1} \quad (1)$$

This equation applies to both simple aqueous solutions and reverse micelles where ( $\rho_1$ ) is the density of the reference micellar solution, ( $\rho$ ) the density of the protein micellar solution, and  $c_p$  the protein concentration.

The experimental measurements of the protein micellar solution density ( $\rho$ ) and the sound velocity ( $u$ ) allows the determination of the solution adiabatic compressibility, using Laplace's equation:

$$\beta = \frac{1}{\rho u^2} \quad (2)$$

The adiabatic compressibility of the reference micellar solution becomes:

$$\beta_1 = \frac{1}{\rho_1 u_1^2} \quad (3)$$

where  $u_1$  is the velocity of the reference micellar solution

In this work we make use of the effective medium theory (Ye et al., 1991) to account for the behavior of reverse micelles, because the relevant acoustic wavelength is always orders of magnitude larger than the micelle size. We take one phase to be the continuum fluid, and the second phase consists of micellar inclusions randomly embedded in the continuous phase. Each of the constituent phases is described by parameters corresponding to the pure phase. We then describe each of the constituent components as a function of the relative volumes of the constituent phases in terms of several parameters.

We can thus relate the micellar protein solution compressibility ( $\beta$ ) to both the micellar ( $\beta_1$ ) and protein ( $\beta_p$ ) compressibilities:

$$\beta = \beta_1(1 - \phi_p) + \beta_p \phi_p \quad (4)$$

$\beta$ ,  $\beta_1$ , and  $\phi_p$  are obtained from  $\rho$ ,  $\rho_1$ ,  $c_p$ ,  $u$  and  $u_1$  for each value of the water:surfactant molar ratio ( $W_0$ ).

The protein compressibility is obtained by:

$$\beta_p = \beta_1 + \frac{\beta - \beta_1}{c_p \varphi_v} \quad (5)$$

Eq. (5) can be written as a function of the protein apparent specific adiabatic compressibility ( $\varphi_k$ )

$$\beta_p = \frac{\beta_1}{\varphi_v} \left( 2\varphi_v - \frac{1}{\rho_1} - 2[u] \right) = \frac{\varphi_k}{\varphi_v} \quad (6)$$

$$\text{where } [u] = \frac{u - u_1}{u_1 \cdot c_p}.$$

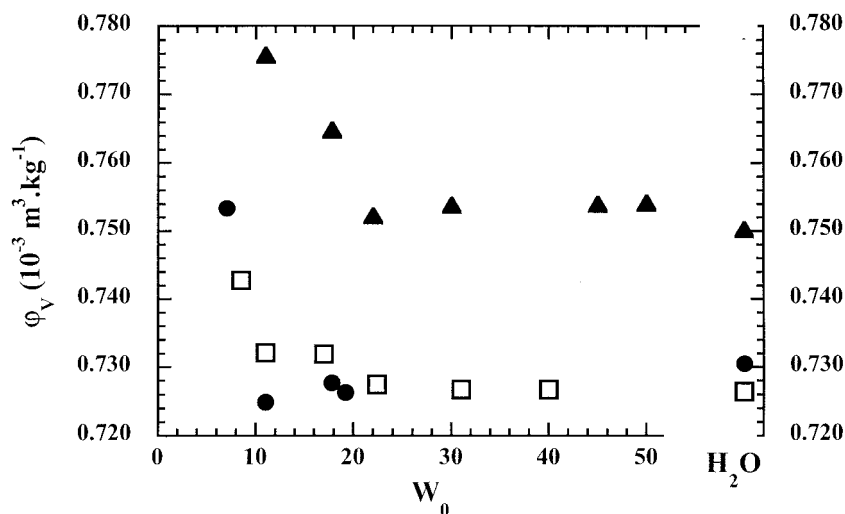
## RESULTS

The reverse micellar system investigated in this work has been previously characterized in a protein-free state by difference ultrasound velocimetry (Amararene et al., 2000). The introduction of proteins into the micellar aqueous core adds an additional complexity to the experiments. Stringent precautions are required to avoid errors in protein concentration that would lead to irreproducible results. As described in the Materials and Methods section, great care has been taken to control and avoid all possible sources of error, such as evaporation, protein insolubility, and precipitation. The difference technique uses as a reference an identical micellar solution devoid of protein. The micelle occupancy by the protein implies a fraction of protein-free micelles. For example, at  $W_0 = 5$  the ratio of free to filled micelles is  $\sim 5$  for a concentration of 3 mg per ml. This rules out the presence of more than one protein molecule per micelle or the existence of protein aggregates. Nevertheless, the existence of protein-free micelles does not interfere with our measurements since the values obtained for such micelles will cancel out by difference. Thus, considering the various caveats discussed in the Materials and Methods section, our data characterize solely the protein, including its interactions. Table 1 summarizes our results: the experimental errors are of the same order of magnitude as those reported in the literature.

## Cytochrome c

This small (13 kDa) basic ( $pI = 10.5$ ) protein has been studied in detail, and the crystal structure of ferricytochrome *c* is known at high resolution (Bushnell et al., 1990). Membrane-bound cytochrome *c* has also been thoroughly studied (Pinheiro and Watts, 1994; Cortese et al., 1998). Depending on the microenvironment, cytochrome *c* appears to adopt a number of conformational states, for example, the mem-

FIGURE 1 Plot of the apparent specific volume  $\phi_v$  of cytochrome *c* (●), lysozyme (□) and  $\beta$ -lactoglobulin (▲) as a function of  $W_0$ , the water:surfactant molar ratio (AOT is 0.1 M in isooctane). The volume scale and the data points on the far right of the figure correspond to values obtained in pure water. Each experiment was carried out at least five times and the data averaged. Experimental errors are shown in Table 1.



brane anionic environment perturbs the native structure of cytochrome *c*, leading to molten globule-like states (Bychkova et al., 1996; Pinheiro et al., 1997). In such states the absence of the characteristic absorbance band at 695 nm is paralleled with the disruption of Met-80 ligation to the iron atom (de Jongh et al., 1995). In AOT reverse micelles, identical spectral characteristics have been reported by Brochette et al. (1988) at high water content. The authors have interpreted this result by the opening of the cytochrome *c* heme crevice under the intense electrostatic field generated by the membrane anionic polar headgroups (Pinheiro et al., 1997). We have carried out experiments in water and reverse micelles in the 8.2 to 19.2  $W_0$  range.

## Volume

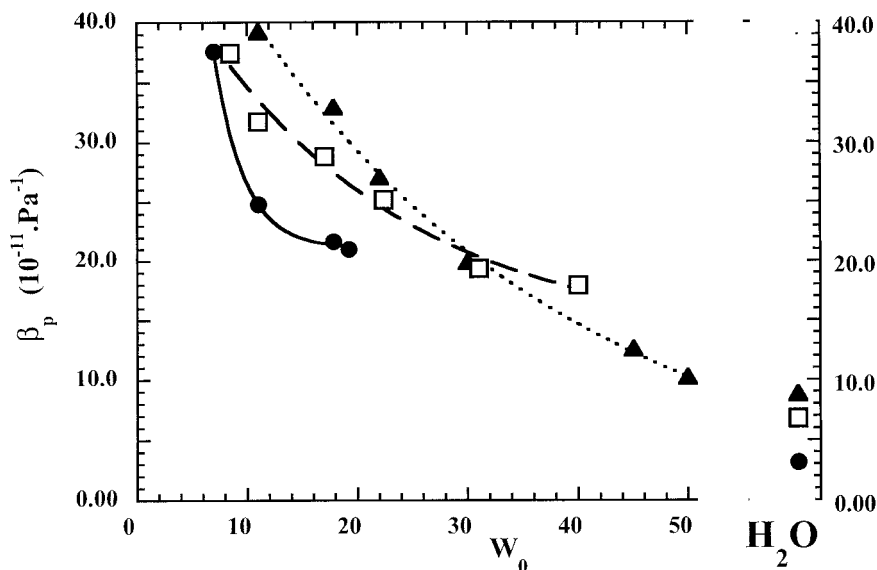
In water, the native protein displays a partial specific volume of  $0.730 \pm 0.002 \text{ ml} \cdot \text{g}^{-1}$ , in agreement with the

literature (Chalikian et al., 1995). In highly hydrated reverse micelles ( $W_0 = 17.8$  and  $19.2$ ) the micellar microenvironment does not seem to affect the specific volume much, since its absolute value is still  $0.726 \pm 0.003 \text{ ml} \cdot \text{g}^{-1}$ . When the water amount decreases to  $W_0 = 11$ , we still find a value of  $0.725 \text{ ml} \cdot \text{g}^{-1}$ . However, as hydration decreases further from  $W_0 = 11$  to  $8$ , the volume increases abruptly to  $0.753 \text{ ml} \cdot \text{g}^{-1}$ . It is remarkable that such a volume jump is observed for a rather small change in  $W_0$  (Fig. 1).

## Compressibility

For the native protein in water, we find a compressibility  $\beta = 3 \pm 0.5 \times 10^{-11} \text{ Pa}^{-1}$  ( $\phi_k = 2.2 \times 10^{-11} \text{ Pa}^{-1} \cdot \text{ml} \cdot \text{g}^{-1}$ ), in the same range as Chalikian et al. (1996). In reverse micelles, at the lowest water content ( $W_0 = 8$ ), we obtain a high value for adiabatic compressibility,

FIGURE 2 Plot of the adiabatic compressibility  $\beta$  of cytochrome *c* (●), lysozyme (□) and  $\beta$ -lactoglobulin (▲) as a function of  $W_0$ . On the far right, values obtained in pure water are shown. The experimental conditions are the same as in Fig. 1.





**TABLE 1** Apparent volumes  $\varphi_v$  and adiabatic compressibilities  $\beta$  of cytochrome *c*, lysozyme, MBP, and  $\beta$ -lactoglobulin as a function of  $W_0$

$W_0^*$	Cytochrome <i>c</i>		Lysozyme		MBP		$\beta$ -Lactoglobulin	
	$\varphi_v$	$\beta$	$\varphi_v$	$\beta$	$\varphi_v$	$\beta$	$\varphi_v$	$\beta$
5.6					0.731	35		
8.2	0.753	38	0.743	37	0.735	36		
11	0.725	25	0.732	32			0.776	39
17.8	0.728	22	0.732	29	0.732	33	0.765	33
19.2	0.726	21						
22.4			0.727	25	0.731	33	0.752	27
30			0.727	19			0.754	20
40			0.727	18				
45							0.754	13
50							0.754	10
H <sub>2</sub> O	0.730	3	0.726	7	0.725	−1	0.750	9

Apparent volumes are expressed in  $\text{ml} \cdot \text{g}^{-1}$ , the maximum error is  $\pm 0.003 \text{ ml} \cdot \text{g}^{-1}$ . Adiabatic compressibilities are expressed in  $10^{-11} \text{ Pa}^{-1}$ , the maximum error is  $\pm 2 \times 10^{-11} \text{ Pa}^{-1}$ .

\*In reverse micelles of 0.1 M AOT in isooctane.

†In reverse micelles of 0.15 M tetraethylene glycol monododecyl ether ( $\text{C}_{12}\text{E}_4$ ) in decane  $W_0 = 22.4$ ; compressibility values for cytochrome *c* and MBP are:  $\beta = 4 \times 10^{-11} \text{ Pa}^{-1}$  and  $2 \times 10^{-11} \text{ Pa}^{-1}$ , respectively.

$\beta = 37 \times 10^{-11} \text{ Pa}^{-1}$  ( $\varphi_k = 28 \pm 2 \times 10^{-11} \text{ Pa}^{-1} \cdot \text{ml} \cdot \text{g}^{-1}$ ). By increasing hydration to  $W_0 = 11, 17.8$ , and, finally, 19.2 (the protein upper solubility limit), the compressibility decreases to  $21 \pm 2 \times 10^{-11} \text{ Pa}^{-1}$  (Fig. 2). In contrast, in nonionic micelles of  $\text{C}_{12}\text{E}_4$  (Merdas et al., 1996), where electrostatic interactions are offset, the compressibility  $\beta$  drops to  $4.0 \times 10^{-11} \text{ Pa}^{-1}$  at  $W_0 = 22$ , close to the value obtained in bulk water. At the same time, we observe the full restoration of the visible absorbance band at 695 nm, implying the religation of Met-80 by heme (spectrum not shown).

Lysozyme

The structure of lysozyme ( $\text{pI} = 10$ ), an enzyme of 14.5 kDa, is also well documented (Imoto et al., 1972). Its behavior in reverse micelles has been described by Grandi et al. (1981). Like other basic proteins, its conformation may be affected by the electrostatic field generated by the anionic surfactant polar headgroups and the protein positive charges. We carried out measurements on lysozyme in water and in reverse micelles in the  $W_0 = 8$  to 40 range.

Volume

In bulk water we find a value of  $0.726 \pm 0.002 \text{ ml} \cdot \text{g}^{-1}$  for the apparent specific volume, in good agreement with the literature (Sasahara et al., 1999). In AOT reverse micelles, at the highest hydration level measured ( $W_0 = 40$ ), its value of  $0.727 \pm 0.003 \text{ ml} \cdot \text{g}^{-1}$  is similar to that measured in water and remains constant down to  $W_0 = 17.8$  (Fig. 1). The specific volume then increases progressively as the amount of water present decreases, reaching a value of  $0.743 \text{ ml} \cdot \text{g}^{-1}$ , at the lowest  $W_0$  measured (8.2).

Compressibility

In water, we have obtained a value for  $\beta$  of  $6.8 \times 10^{-11} \text{ Pa}^{-1}$ , in agreement with published data (Kharakoz and Sarvazyan, 1993; Priev et al., 1996). In AOT reverse micelles we observe a progressive decrease of the adiabatic compressibility as a function of increasing hydration. At  $W_0 = 8.2$ ,  $\beta = 37 \times 10^{-11} \text{ Pa}^{-1}$  (i. e. of the same order as cytochrome *c*) and decreases progressively until  $W_0 = 40$ , where it reaches  $18 \times 10^{-11} \text{ Pa}^{-1}$ . It always remains higher than in bulk water (Fig. 2), similar to cytochrome *c*.

$\beta$ -Lactoglobulin

The major whey protein of the milk of ruminants and other mammals has an isoelectric point of 5.1. Its structure has been solved at 1.8 Å resolution (Brownlow et al., 1997). It is a predominantly  $\beta$ -sheet protein, consisting of a  $\beta$ -barrel made of eight antiparallel  $\beta$ -strands. At neutral pH, the protein exists as a dimer of about 35 kDa. It dissociates into two monomers at pH 2.0, retaining the same x-ray structure as at neutral pH (Kuwata et al., 1999). We have taken advantage of the this unusual conformational property to investigate the volume and compressibility changes of the protein first in water, then upon subunit dissociation at pH 2.0, and finally in AOT reverse micelles between  $W_0$  values of 11 and 50 (Figs. 1 and 2).

Volume

In water, the partial specific volume found in literature for  $\beta$ -lactoglobulin is quite variable in the 0.734–0.751  $\text{ml} \cdot \text{g}^{-1}$  range, probably due to different experimental conditions. In this work, we have obtained a value of 0.750

$\text{ml} \cdot \text{g}^{-1}$ , in good agreement with Gekko and Hasegawa (1986). In 0.01 M HCl at pH 2.0, as the dimer protein dissociates into two subunits, it exposes to the solvent a surface of  $568 \text{ \AA}^2$  per monomer (Brownlon et al., 1997) and its volume increases to  $0.770 \text{ ml} \cdot \text{g}^{-1}$ . The uncorrected difference corresponds to a value of  $370 \text{ ml/mole}$  of protein. However, after correction for carboxyl and histidine protonation (Foygel et al., 1995), we obtain a difference of only  $61 \text{ ml/mole}$  of protein, close to experimental error.

Turning now to reverse micelles at the highest water content studied in this work ( $W_0 = 50$ ), we find a volume of  $0.754 \pm 0.003 \text{ ml} \cdot \text{g}^{-1}$ , similar to the aqueous solution value. Furthermore, it remains unchanged at  $W_0$  values of 30 and 22.4. At  $W_0 = 11$ , the volume increases to  $0.776 \text{ ml} \cdot \text{g}^{-1}$ , in the same manner as for the other proteins studied. Note that in AOT reverse micelles,  $\beta$ -lactoglobulin cannot be solubilized at lower  $W_0$  values. This fact is probably due to its relatively low isoelectric point ( $\text{pI} = 5.1$ ) and to its size.

### Compressibility

In water, we obtain a value of  $\beta = 8.8 \times 10^{-11} \text{ Pa}^{-1}$  for the dimer, in agreement with the literature (Gekko and Hasegawa, 1986; Kharakoz and Sarvazyan, 1993; Priev et al., 1996). Note that in 0.01 M HCl at pH 2.0,  $\beta$  increases to  $12 \times 10^{-11} \text{ Pa}^{-1}$  for the monomeric protein. Thus, upon dimer dissociation, a small increase in volume parallels a larger increase in compressibility. At  $W_0 = 11$ , its compressibility is the highest measured in this study:  $\beta = 39 \times 10^{-11} \text{ Pa}^{-1}$ . It progressively decreases as a function of hydration, although the curve profile (Fig. 2) is obviously different from the other basic proteins studied. At the highest  $W_0$  value (50), the protein compressibility decreases to  $10 \times 10^{-11} \text{ Pa}^{-1}$ , and the curve can be extrapolated to the value obtained in aqueous solvents.  $\beta$ -lactoglobulin can thus serve as a reference for comparison with the basic proteins.

### MBP

MBP is essential for the formation of the myelin sheath. Although the small (18.5 kDa) peripheral membrane basic ( $\text{pI} = 10.6$ ) protein has been completely sequenced, its three-dimensional structure in native myelin is not known. In dilute aqueous solutions, the protein, devoid of disulfide bridges, displays a high degree of conformational flexibility (Liebes et al., 1975). The molecular volume of the protein doubles upon hydration, from  $22,000 \text{ \AA}^3$  to  $47,300 \text{ \AA}^3$  (Martenson, 1978) evidencing extensive solvation. We have shown previously that in AOT reverse micelles MBP refolds from an unordered structure to a stable, ordered conformation, much less prone to proteolysis than the flexible aqueous form (Nicot et al., 1993). The micellar cavity thus

provides MBP with a chaperonin-like microenvironment, where micelle-assisted folding can proceed in the absence of aggregation (Nicot and Waks, 1995).

### Volume

We have found for the partial specific volume of MBP in water a value of  $0.725 \pm 0.002 \text{ ml} \cdot \text{g}^{-1}$ , in agreement with the value of  $0.720 \text{ ml} \cdot \text{g}^{-1}$  reported by Liebes et al. (1975) from the amino acid composition. There is no information available concerning the actual value of the apparent partial specific volume of refolded MBP in its native, membrane-bound form in myelin. We determined the volume of the refolded protein in the aqueous core of AOT reverse micelles as a function of hydration. At the highest water content measured ( $W_0 = 22.4$ ), its value increases slightly from 0.725 in water to  $0.731 \pm 0.003 \times 10^{-3} \text{ ml} \cdot \text{g}^{-1}$ , indeed a very modest variation taking into account experimental errors. Moreover, at all other  $W_0$  values measured (17.8, 8.2, and 5.6) the volume remains almost constant (Fig. 3). It is clear that in reverse micelles the volume of MBP is independent of the amount of water present.

### Compressibility

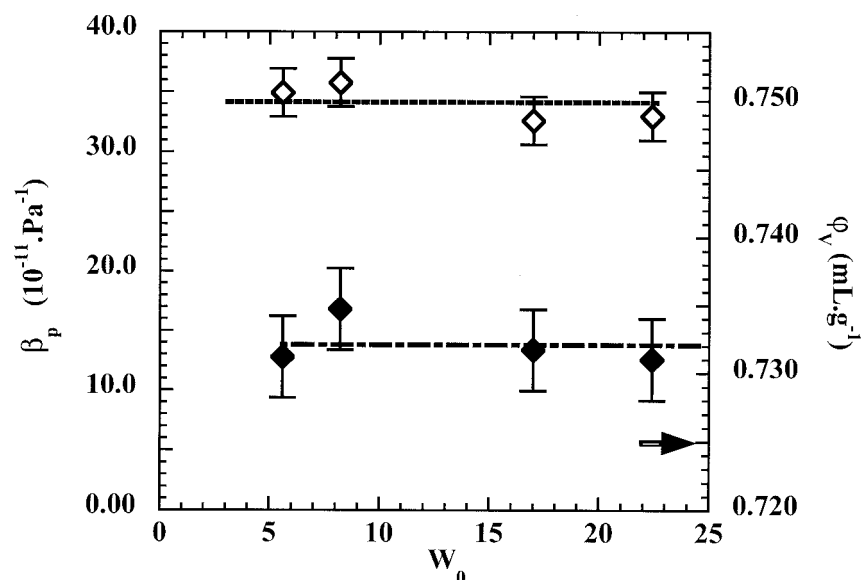
We obtain for the adiabatic compressibility of MBP in pure water a value of  $\beta = -1.5 \times 10^{-11} \text{ Pa}^{-1}$  ( $\varphi_k = -1.0 \times 10^{-11} \text{ Pa}^{-1} \cdot \text{ml} \cdot \text{g}^{-1}$ ). In contrast, at the lowest  $W_0$  value measured (5.6) in AOT reverse micelles, the compressibility obtained is rather high:  $36 \pm 2 \times 10^{-11} \text{ Pa}^{-1}$ . It remains constant regardless of the amount of water present up to  $W_0 = 22.4$ , similar to its apparent specific volume (Fig. 3). This unusual behavior, specific to MBP, will be discussed below. It is interesting that at  $W_0 = 22.4$ , in the nonionic ( $\text{C}_{12}\text{E}_4$ ) reverses micelles devoid of electrostatic interactions (although other interactions remain operative), we find  $\beta = 2.0 \times 10^{-11} \text{ Pa}^{-1}$  ( $\varphi_k = 1.5 \times 10^{-11} \text{ Pa}^{-1} \cdot \text{ml} \cdot \text{g}^{-1}$ ). The latter value being in the same range as that found for native cytochrome *c* in water (see Table 1).

### DISCUSSION

We present in this work novel experimental results obtained by densitometry and sound velocimetry for proteins in both aqueous solvents and protein-sized reverse micelles. Our aim was to correlate the volumetric measurements with increasing levels of controlled hydration. At the same time this strategy has provided useful volumetric data for a protein, MBP, in both the refolded and unfolded conformation without the use of chemical denaturing agents.

We are fully aware that, in addition to hydration, a number of parameters might contribute to the behavior of proteins in such a complex system. For example, the strong electrostatic field present in AOT reverse micelles results in

FIGURE 3 Plot of the adiabatic compressibility  $\beta$  (left  $\diamond$ ) and apparent specific volume  $\phi_v$  (right  $\blacklozenge$ ) of myelin basic protein as a function of the water content of the system,  $W_0$ . The arrow on the far right indicates the volume of MBP in water. The compressibility  $\beta$  of the protein in water is slightly negative and out of scale (see Table 1).



extensive interactions between the basic proteins and the micellar inner wall. This effect leads to the expulsion of electrostricted water and the concentration of counterions, contributing to increase both protein volume and compressibility. The ion solvation and solvent reorganization in the vicinity of the surfactant-solute interface must also be mentioned. The reduced space available within the micelle may induce a perturbation of the chemical potential of macromolecules (Minton, 1992). The anomalous volume and compressibility values of micellar water in both free and bound states, as compared to bulk water (Amararene et al., 2000), can make hydration data difficult to interpret. We discuss these below.

## Volume

The comparison of the variation in apparent specific volume of cytochrome *c*, lysozyme, and  $\beta$ -lactoglobulin as a function of  $W_0$  (Fig. 1) reveals a very similar profile for the three proteins. At high water content, all these proteins display apparent volumes similar to those found in bulk water. This result clearly indicates that the complex micellar system does not affect volumetric parameters of these proteins. At the lowest  $W_0$  value compatible with full protein solubilization, in the 8 to 11  $W_0$  range, the apparent specific volumes reach their highest value for all the proteins examined. Because of the compensating mechanisms operative in volume measurements, this result may be difficult to interpret. It is also clear that protein melting and aggregation can be excluded, as well as dehydration of the uncharged surface. Nevertheless, recall that each polar AOT headgroup binds  $\sim 7$  to 10 water molecules very tightly, leaving very little water available for the protein, which then has to compete for it. Under these conditions (i.e., the absence of

free water), full protein hydration within micelles is ruled out. It is interesting that the volume values ( $0.743$  to  $0.776 \text{ mL} \cdot \text{g}^{-1}$ ) obtained at low  $W_0$  are of the same order of magnitude as the partial specific volume of an average globular protein interior ( $0.764 \text{ mL} \cdot \text{g}^{-1}$ ), based on x-ray coordinate data for 12 globular proteins (Richards, 1977).

## Compressibility

All the proteins examined at the lowest water content (in the 5.6 to 10  $W_0$  range), display the highest compressibility values ( $35$  to  $39 \times 10^{-11} \text{ Pa}^{-1}$ ). It is remarkable that the results obtained fall within the same order of magnitude for all the proteins investigated, including  $\beta$ -lactoglobulin. They are independent of their amino acid sequence, isoelectric point, molecular weight, and conformation. In fact, the compressibility of the protein interior would not be expected to vary greatly because the interior packing density of normal globular proteins falls within a narrow range (Richards, 1977).

As discussed in the preceding paragraph, at  $W_0$  values around 10 or less, the amount of water present is not sufficient to solvate both the surfactant polar headgroups and the protein, which remain poorly hydrated. Under these experimental conditions, do we experimentally measure intrinsic protein compressibility? The value found in this report is somewhat higher than the empirical estimates proposed by Kharakoz and Sarvazyan (1993) or Chalikian et al. (1995). The difference may be due to strong interactions occurring between a protein in close contact with a number of non-solvated surfactant polar headgroups. The value found is thus probably the sum of two parameters: one due to the interior of the barely-hydrated protein and a second one originating from interactions with the dry polar

headgroups interacting with sites on the protein surface. At this point, it seems difficult to estimate their respective contributions.

When the amount of water increases, we observe, as expected, a progressive decrease of adiabatic compressibility (Fig. 2). There are, however, obvious differences between the basic proteins and  $\beta$ -lactoglobulin. The compressibility profile of the latter declines smoothly, and can be extrapolated to its value in pure water. The compressibility values obtained in this work between the two extreme  $W_0$  values (10 and 50) reflect indeed the actual hydration status of  $\beta$ -lactoglobulin in reverse micelles and validate our experimental strategy.

Lysozyme and cytochrome *c* compressibilities, on the other hand, seem to level off at much higher values than those obtained in aqueous solvents. This effect, which is not observed in  $\beta$ -lactoglobulin, can be attributed to electrostatic interactions between the negatively charged surfactant polar headgroups and the positively charged proteins, leading to a reduced amount of electrostricted water and protein structural perturbations. Such an interpretation is confirmed by the low values of  $\beta$  found in nonionic reverse micelles in the range of aqueous values, in parallel with the recovered heme ligation of cytochrome *c* and native conformation. Yet, we observe differences between the two basic proteins, probably due to the extent of their structural alteration in AOT reverse micelles. Although cytochrome *c* exhibits a sharp compressibility transition between  $W_0$  values 8 and 11 (Fig. 2), which appears to indicate a substantial structural disruption with the possibility of a molten globule-like state (Bychkova et al., 1996; Pinheiro et al., 1997), the relative conformational perturbation of lysozyme is obviously less extensive. Its four disulfide bonds act as structural stabilizers, whereas cytochrome *c* has no disulfides.

### The folding transition of MBP

The apparent specific volume and compressibility of denatured, unfolded proteins and peptides have been previously investigated by exposing the solutes to chemical additives (urea or guanidinium hydrochloride) and by extrapolating the denaturant concentration at infinite dilution (Tamura and Gekko, 1995). However, the exact nature of the molecular interaction between the peptide chain and the denaturing agents is not well understood. One of the proposed mechanisms suggests a preferential binding of the denaturants to the polypeptide chains (Lee and Timasheff, 1974). To avoid these drawbacks, we have carried out experiments with unfolded MBP in water free of denaturing agents, as well as in reverse micelles where MBP refolds.

Our results show that the transition between the two forms of MBP are accompanied by a very small volume variation ( $0.006 \pm 0.003 \text{ ml} \cdot \text{g}^{-1}$ ). As is indeed reported in literature (Chalikian and Breslauer, 1996; Vidugiris and Royer, 1998; Sasahara et al., 1999), there is very little or no

volume difference between the aqueous unfolded conformation, and the compact refolded state of proteins. This result has been referred to as the protein volume paradox, and has been interpreted by Chalikian and Breslauer (1996) on the basis of the thermal volume concept, which is related to volume changes for protein unfolding involving changes in the solvent-accessible surface area. Although our data show a very modest change in MBP volume, we cannot rule out that volume effects, absent in bulk aqueous solutions, may exist within the electrostatic field of reverse micelles, for example, due to an increase in the number and/or size of protein voids.

The unfolded form of MBP displays a small negative value of adiabatic compressibility in pure water, but less than the value estimated by using the additivity scheme proposed by Kharakoz (1997) for fully extended oligopeptides and polypeptides. However, since it is known that a residual  $\beta$ -pleated sheet and hydrophobic cluster persists in the water-unfolded form of MBP (Martenson, 1986), the result is not inconsistent with the above empirical estimation. It is also in agreement with recent studies concluding that the unfolded peptide state, even in 6 M guanidinium hydrochloride, is not modeled by a random-coil polypeptide chain (Dill and Shortle, 1991).

In contrast to all other proteins studied in this report, the myelin protein displays constant apparent volume and compressibility values, independent of the water concentration (Fig. 3) and thus of hydration. This effect results from the tight and extensive contacts of the protein adsorbed on the micellar wall as observed on lipid membranes (Mueller et al., 2000), with probable expulsion of interfacial water. We know, on the other hand, that the final MBP micellar conformation (20%  $\alpha$ -helix, 40%  $\beta$ -sheet) is unaffected by the water amount present (Nicot et al., 1985) in the 3 to 30  $W_0$  range. The protein thus displays a form of a frozen structure in reverse micelles (Waks and Beychok, 1974), which is also observed by time-resolved fluorescence experiments (Nicot et al., 1985). The volumetric properties of MBP in reverse micelles thus reflect a different aspect of the protein specific structural features.

We believe that information obtained in this work may prove important for the construction of de novo stable proteins, in which correct chain packing is crucial (Harbury et al., 1998). The recent emergence of misfolding related diseases, linked to neurodegeneration (Aguzzi, 1998; Cohen, 1999) constitutes another example of the growing importance of an in depth understanding of the correct protein packing and folding. Further investigation of the volumetric properties that characterize the correct folding of proteins obtained from genetic engineering should also improve our understanding of the structural properties of peptides used as therapeutic agents. For example, it has been recently reported that  $\beta$ -interferon administered to multiple sclerosis patients may induce lethal complications (Du-



relli et al., 1998), probably resulting from incomplete folding and/or poor packing.

We thank Dr. A. Amararene for his contribution to the early stages of this work. We also express our gratitude to Dr. C. Nicot, and thank Dr. P. C. Kahn and Dr. K. Waters for the careful reading of the manuscript.

This work was supported in part by the Centre National de la Recherche Scientifique (Physique et Chimie du Vivant) and a grant from the Simone and Cino Del Duca Foundation.

## REFERENCES

- Aguzzi, A. 1998. Protein conformation dictates prion strain. *Nat. Med.* 4:1125–1126.
- Amararene, A., M. Gindre, J.-Y. Le Huérou, W. Urbach, D. Valdez, and M. Waks. 2000. Adiabatic compressibility of AOT [sodium bis(2-ethylhexyl)sulfosuccinate] reverse micelles: analysis of a simple model based on micellar size and volumetric measurements. *Physiol. Rev. E.* 61:682–689.
- Breazaele, A., J. H. Cantrell, and J. S. Heyman. 1981. Ultrasonic wave velocity and attenuation measurements. In *Ultrasonics*, Vol. 19. P. D. Edmonds, editor. Academic Press, New York. 67–135.
- Brochette, P., C. Petit, and M. P. Pileni. 1988. Cytochrome *c* in sodium bis 2-(ethylhexyl) sulfosuccinate reverse micelles: structure and reactivity. *J. Phys. Chem.* 92:3505–3511.
- Brownlow, S., J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polcarpov, A. C., T. North, and L. Sawyer. 1997. Bovine  $\beta$ -lactoglobulin at 1.8 Å resolution—still an enigmatic lipocain. *Structure.* 5:481–495.
- Bushnell, G. W., G. V. Louie, and G. D. Bayer. 1990. High resolution three dimensional structure of horse heart cytochrome *c*. *J. Mol. Biol.* 214: 585–595.
- Bychkova, V. E., A. E. Dujsekina, S. I. Klenin, E. I. Tiktopulo, V. N. Uversky, and O. B. Ptitsyn. 1996. Molten globule-like state of cytochrome *c*. *Biochemistry.* 35:6058–6063.
- Chalikian, T. V., and K. Breslauer. 1996. On volume changes accompanying conformational transitions in biopolymers. *Biopolymers.* 39: 619–626.
- Chalikian, T. V., V. S. Gindkin, and K. J. Breslauer. 1995. Volumetric characterizations of the native, molten globule and unfolded states of cytochrome *c* at acidic pH. *J. Mol. Biol.* 250:291–306.
- Chalikian, T. V., M. Totrov, R. Abagayan, and K. J. Breslauer. 1996. The hydration of globular proteins as derived from volume and compressibility measurements: cross correlating thermodynamic and structural data. *J. Mol. Biol.* 260:588–603.
- Cohen, F. E. 1999. Protein misfolding and prion diseases. *J. Mol. Biol.* 293:313–320.
- Cortese, J. D., A. L. Voglino, and C. R. Hackenbrock. 1998. Multiple conformations of physiological membrane-bound cytochrome *c*. *Biochemistry.* 37:6402–6409.
- de Jongh, H. H. J., T. Ritsema, and J. A. Killian. 1995. Lipid specificity for membrane mediated partial unfolding of cytochrome *c*. *FEBS Lett.* 360:255–260.
- Dill, K. A., and D. Shortle. 1991. Denatured states of proteins. *Annu. Rev. Biochem.* 60:795–825.
- Durelli, L., M. R. Bongioanni, B. Ferrero, A. Oggero, A. Marzano, and R. Rizzetto. 1998. Interferon treatment for multiple sclerosis: autoimmune complications may be lethal. *Neurology.* 50:570.
- Foygel, K., S. Spector, S. Chatterjee, and P. C. Kahn. 1995. Volume changes of the molten globule transition of horse heart ferricytochrome *c*: a thermodynamic cycle. *Protein Sci.* 4:1426–1429.
- Gavish, B., E. Gratton, and C. J. Hardy. 1983. Adiabatic compressibility of globular proteins. *Proc. Natl. Acad. Sci. U.S.A.* 80:750–754.
- Gekko, K., and Y. Hasegawa. 1986. Compressibility-structure relationship of globular proteins. *Biochemistry.* 25:6563–6571.
- Gekko, K., and H. Noguchi. 1979. Compressibility of proteins at 25°C. *J. Phys. Chem.* 83:2706–2714.
- Grandi, C., R. E. Smith, and P. L. Luisi. 1981. Micellar solubilization of biopolymers in organic solvents. *J. Biol. Chem.* 256:837–843.
- Harbury, P. B., J. J. Plecs, B. Tidor, T. Alber, and P. S. Kim. 1998. High-resolution protein design with backbone freedom. *Science.* 282: 1462–1467.
- Imoto, T. L., N. Johnson, A. C. T. North, D. C. Philips, and J. A. Rupley. 1972. Lysozyme. In *The Enzymes*. P. D. Boyer, editor. Academic Press, New York. 665–808.
- Kharakoz, D. P. 1997. Partial volumes and compressibilities of extended polypeptide chains in aqueous solution: additivity scheme and implication of protein unfolding at normal and high pressure. *Biochemistry.* 23:10276–10285.
- Kharakoz, D. P., and A. P. Sarvazyan. 1993. Hydrational and intrinsic compressibilities of globular proteins. *Biopolymers.* 13:11–26.
- Klapper, M. N. 1971. On the nature of protein interior. *Biochim. Biophys. Acta.* 229:557–566.
- Kuwata, K., M. Hoshino, V. Forge, S. Era, A. Batt, and Y. Goto. 1999. Solution structure and dynamics of bovine  $\beta$ -lactoglobulin. *Protein. Sci.* 8:2541–2545.
- Lee, J. C., and S. N. Timasheff. 1974. Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride. *Biochemistry.* 13:257–265.
- Liebes, L. F., R. Zand, and W. D. Phillips. 1975. Solution behavior, circular dichroism and 220MHz PMR studies of the bovine myelin basic protein. *Biochim. Biophys. Acta.* 405:27–39.
- Luisi, P. L., and L. J. Magid. 1986. Solubilization of enzymes and nucleic acids in hydrocarbon micellar solutions. *CRC Crit. Rev. Biochem.* 20: 409–474.
- Martenson, R. E. 1978. The use of gel filtration to follow conformational changes in proteins: conformational flexibility of bovine myelin basic protein. *J. Biol. Chem.* 253:8885–8893.
- Martenson, R. E. 1986. Possible hydrophobic region in myelin basic protein consisting of an orthogonally packed beta sheet. *J. Neurochem.* 46:1612–1622.
- Merdas, A., M. Gindre, R. Ober, C. Nicot, W. Urbach, and M. Waks. 1996. Nonionic surfactant reverse micelles of C<sub>12</sub>E<sub>4</sub> in dodecane: temperature dependence of size and shape. *J. Phys. Chem.* 100:15180–15186.
- Minton, A. P. 1992. Confinement as a determinant of macromolecular structure and reactivity. *Biophys. J.* 63:1090–1100.
- Mueller, H., H.-J. Buff, and E. Bamberg. 2000. Adsorption of membrane-associated proteins to lipid bilayers studied with an atomic force microscope: myelin basic protein and cytochrome *c*. *J. Phys. Chem.* 104:4552–4559.
- Nicot, C., M. Vacher, L. Denoroy, P. C. Kahn, and M. Waks. 1993. Limited proteolysis of myelin basic protein in a system mimetic of the myelin interlamellar aqueous spaces. *J. Neurochem.* 60: 1283–1291.
- Nicot, C., M. Vacher, M. Vincent, J. Gallay, and M. Waks. 1985. Membrane proteins in reverse micelles: myelin basic protein in a membrane-mimetic environment. *Biochemistry.* 24:7024–7032.
- Nicot, C., and M. Waks. 1995. Proteins as invited guests of reverse micelles: conformational effects, significance, applications. *Biotechnol. Genet. Engin. Revs.* 13:267–314.
- Oldfield, C. 1994. Enzymes in water-in-oil microemulsions (reversed micelles): principles and applications. *Biotechnol. Genet. Engin. Rev.* 12:255–327.
- Paci, E., and B. Velikson. 1997. On the volume of macromolecules. *Biopolymers.* 41:785–797.
- Pinheiro, T. J. T., G. A. Elöve, A. Watts, and H. Roder. 1997. Structural and kinetic description of cytochrome *c* unfolding induced by the interaction with lipid vesicles. *Biochemistry.* 36:13122–13132.
- Pinheiro, T. J. T., and A. Watts. 1994. Lipid specificity in the interaction of cytochrome *c* with anionic phospholipid bilayers revealed by solid-state <sup>31</sup>P NMR. *Biochemistry.* 33:2451–2458.

- Priev, A., A. Almagor, S. Yedgar, and B. Gavish. 1996. Glycerol decreases the volume and compressibility of protein interior. *Biochemistry*. 35: 2061–2066.
- Rashin, A. A., M. Iofin, and B. Honig. 1986. Internal cavities and buried waters in globular proteins. *Biochemistry*. 23:3619–3625.
- Richards, F. M. 1977. Areas, volumes, packing and protein structure. *Annu. Rev. Biophys. Bioengin.* 6:151–176.
- Richards, F. M., and W. A. Lim. 1994. An analysis of packing in the protein folding problem. *Q. Rev. Biophys.* 26:4.: 423–498.
- Sarvazyan, A. P. 1982. Developments of methods of precise ultrasonic measurements in small volumes of liquids. *Ultrasonics*. 20:151–154.
- Sarvazyan, A. P. 1991. Ultrasonic velocimetry of biological compounds. *Annu. Rev. Biophys. Biophys. Chem.* 20:321–342.
- Sasahara, K., M. Sakurai, and K. Nitta. 1999. The volume and compressibility changes of lysozyme associated with guanidinium chloride and pressure-assisted unfolding. *J. Mol. Biol.* 291:693–701.
- Takano, K., J. Funahashi, Y. Yamagata, S. Fujii, and K. Yutani. 1997. Contribution of water molecules in the interior of a protein to the conformational stability. *J. Mol. Biol.* 274:132–142.
- Tamura, Y., and K. Gekko. 1995. Compactness of thermally and chemically denatured ribonuclease A as revealed by volume and compressibility. *Biochemistry*. 34:1878–1884.
- Vidugiris, G. J. A., and C. Royer. 1998. Determination of the volume changes for pressure-induced transitions of apomyoglobin between the native, molten globule, and unfolded state. *Biophys. J.* 75: 463–470.
- Waks, M. 1986. Proteins and peptides in water-restricted environments. *Proteins*. 1:4–15.
- Waks, M., and S. Beychok. 1974. Induced conformational states in human apohemoglobin on binding haptoglobin 1–1. Effect of added heme as a probe of frozen structures. *Biochemistry*. 113:15–21.
- Ye, L., D. A. Weitz, J. Liu, P. Sheng, and J. S. Huang. 1991. Sound propagation in sodium di-2ethyl-hexylsulfosuccinate micelles and microemulsions. *Phys. Rev. A*. 44:8249–8263.